C

Appl Microbiol Biotechnol (1987) 26: 130–135

Applied and Microbiology Biotechnology

Phosphate repression of cephamycin and clavulanic acid production by *Streptomyces clavuligerus*

Ahmed Lebrihi, Pierre Germain, and Gérard Lefebvre

Laboratoire de Microbiologie Industrielle, E.N.S.A.I.A., Institut National Polytechnique de Lorraine, Nancy, France

Summary. Production of cephamycin and clavulanic acid by *Streptomyces clavuligerus* is controlled by the phosphate concentration. Phosphate represses the biosynthesis of "cephamycin synthetase", expandase and "clavulanic acid synthetase". In the presence of 2 mM phosphate, the specific activities of expandase, "cephamycin synthetase" and "clavulanic acid synthetase" were higher than in the presence of 75 mM phosphate. The specific activity of cephamycin synthetase is maximal with an initial phosphate concentration of 10 mM, whereas the specific activity of expandase is maximal with 1 mM phosphate. A correlation between cephamycin synthetase specific activity and expandase specific activity was established at phosphate concentrations higher than 10 mM. This shows that the expandase is an important enzyme in the mechanism by which the phosphate concentration affects the biosynthesis of cephamycin.

Introduction

Streptomyces clavuligerus, first described by Higgens and Kastner (1971), produces four β-lactam antibiotics (Brown et al. 1976; Nagarajan 1972). Two of these are cephalosporins [7-(5-amino-5-carboxy-valeramido)-3-carboxyloxymethyl-3-cephem-4-carboxylic acid and cephamycin c]; penicillin N and clavulanic acid are also produced.

Production of cephamycin and clavulanic acid is controlled by the phosphate content of the medium. Cephamycin production is reduced by 50% when the phosphate concentration in fermentation medium is 50 mM and by 80% when it is 100 mM (Aharonowitz and Demain 1977). Romero et al. (1984) have shown the same inhibition of production of clavulanic acid by *S. clavuligerus*.

Recently, Lubbe et al. (1985) demonstrated that the biosynthesis of expandase was repressed by inorganic phosphate, while the biosynthesis of cyclase and epimerase was not. This paper describes an investigation of the phenomenon of repression of expandase, cephamycin and clavulanic acid synthetases.

Offprint requests to: P. Germain, 2, av. de la Forêt de Haye, F-54500 Vandœuvre-lès-Nancy, France

Materials and methods

Microorganisms. These studies were carried out with *Streptomyces clavuligerus* NRRL 3585. *Escherichia coli* (Esss), a supersensitive strain to β-lactam antibiotics and resistant to streptomycin, was isolated in our laboratory from *E. coli* (Ess 22-35), donated by Demain (Massachusetts Institute of Technology, Cambridge, USA). *Klebsiella pneumonia* ATCC 29665, a penicillin-resistant strain used for the assay of clavulanic acid assay, was donated by Martin (University of Leon, Spain).

Medium and culture conditions. Spores of *S. clavuligerus* were maintained in 20% glycerol at −20° C; 0.1 ml of this suspension was used to inoculate 50 ml of the seed medium and incubated for 48 h. The resulting seed culture was inoculated into the desired fermentation medium (2% v/v). All liquid cultures were conducted in 1000-ml or 3000-ml Erlenmeyer flasks containing 200 ml or 600 ml of medium, respectively, at 28° C, pH 6.9 on a rotatory shaker at 250 rpm. The media were the same as those described by Aharonowitz and Demain (1977); except where mentioned, K_2HPO_4 was used as described in the text and $MgSO_4$ was added at 1 g/l.

Growth measurement. For the determination of dry cell weight (DCW), the following method, devised for *S. clavuligerus* by Brana (personal communication), was used. To 0.5 ml whole broth was added 0.5 ml of 2.5 M HCl. Water (4 ml) was added and the suspension treated by a sonicator (Ultrasonics Inc.; model W 225 R) for 40 s at 60 W; the optical density (OD) was immediately measured at 660 nm with a digital colorimeter

(Chemtrix type 24). The bacterial suspension was diluted above 0.3 OD. The OD units were transformed to DCW by a correlation OD-DCW; 1 g DCW/l corresponds to an OD of 1.8.

Determination of glycerol. Glycerol in the culture broth was determined as described by Bok and Demain (1977).

Clavulanic acid and cephamycin determination. Clavulanic acid was determined by the microbiological method described by Romero et al. (1984) and by high-performance liquid chromatography (HPLC). Cephamycin was determined by microbiological dosage as described by Aharonowitz and Demain (1978).

Preparation of resting cell system (RCS) and cephamycin synthetase assay. The method of Hu et al. (1984) was used. *S. clavuligerus* mycelia were harvested by filtration washed twice with a 10 mM morpholinopropanesulphonic acid (MOPS) buffer solution (pH 6.9, containing 100 mg/l streptomycin) and resuspended in 200 mM MOPS buffer (pH 6.9, containing 200 mg/l streptomycin). To 5 ml of the mycelial suspension in a 25-ml flask, 5 ml water was added. After 6 h of incubation at 28° C, the β-lactams produced and the DCW were measured and the specific activity of cephamycin synthetase calculated. Specific activity was defined as milligrams of cephamycin formed per gram of DCW per hour.

Preparation of crude cell-free extracts. Crude cell-free extracts were prepared according to Brana et al. (1985). Mycelia were recovered from the fermentation broth by filtration, washed twice with distilled water, and suspended in a buffer (50 mM Tris-HCl, pH 7.2, 0.1 mM dithiothreitol, 10 mM $MgSO_4 \cdot 7H_2O$ and 14 mM KCl). The mycelia were disrupted in an ice-water bath using a sonicator (Ultrasonics Inc.; model W 225 R) for 1 min at 50% duty cycle, 120 W. Cell fragments were removed by centrifugation at 10000*g* for 15 min. The supernatant was used as the enzymatic extract. All manipulations were performed at 4° C. The protein concentration of the cell-free extracts was measured by the method of Lowry et al. (1951).

Expandase assay. The 100 µl final reaction mixture contained 50 mM Tris-HCl pH 7.2, 10 mM $MgSO_4 \cdot 7H_2O$, 14 mM KCl, 0.4 mM $FeSO_4$, 0.67 mM ascorbic acid, 0.1 mM dithiothreitol, 0.6 mM α-ketoglutarate, 0.2 mM penicillin N and 85 µl enzyme solution. The reaction was started by the addition of penicillin N and the mixture was incubated at 21° C without agitation. After removal of the precipitated proteins, the supernatant fluid was analysed for deacetoxycephalosporin C (DOC) formation by HPLC (see Jensen et al. 1983). Specific activity was defined as micrograms of DOC formed per milligram of protein per hour.

High-performance liquid chromatography. The equipment used consisted of a 590 pump, U6K injector, 481 variable wavelength detector, 740 data module and a C_{18} column (Novapak).

All equipment was obtained from Waters Scientific. Conditions of analysis and mobile phase were the same as described by Jensen et al. (1983) except that the methanol was suppressed.

Chemicals. Deacetoxycephalosporin C, deacetylcephalosporin C and penicillin N were supplied by Nuech (Ciba-Geigy, Switzerland); clavulanic acid was a gift of Beecham (England); cephalosporin C and other reagents were obtained from Sigma.

Results

Effect of inorganic phosphate on fermentation kinetics

Two fermentations were carried out at two different phosphate concentrations: 2 mM and 75 mM respectively. The initial specific growth rates of *S. clavuligerus* were identical (0.04 h^{-1}).

In fermentation carried out with a phosphate concentration of 2 mM, the specific growth rate fell from 0.04 h^{-1} to 0.006 h^{-1} after 72 h of fermentation. This was a result of reduction in phosphate concentration, which leads to reduced glycerol consumption, whereas the specific rate of clavulanic acid and cephamycin production changed from 0.9 to 1.84 mgh^{-1} g^{-1} DCW when the specific growth rate changed from 0.025 h^{-1} to 0.006 h^{-1} (Fig. 1).

In fermentation carried out with a phosphate concentration of 75 mM, the specific growth rate changed from 0.04 h^{-1} to 0.016 h^{-1} after 72 h of fermentation. This specific growth rate at 72 h is much higher than that in fermentation carried out with a phosphate concentration of 2 mM, which would explain the rapidity of glycerol consumption. Growth stopped after 96 h of fermentation due to exhaustion of glycerol. This shows that under these conditions glycerol is the limiting factor. Biosynthesis of clavulanic acid and cephamycin takes place late with specific rates of production of 0.18 and 0.07 mgh^{-1} g^{-1} DCW respectively when the specific growth rate is 0.016 h^{-1}.

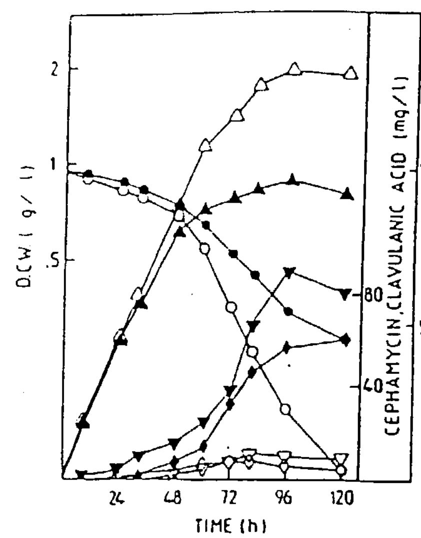

Fig. 1. Growth (▲, △), glycerol consumption (●, ○) and cephamycin (◆, ◇) and clavulanic acid (▼, ▽) biosynthesis in fermentations with 2 mM *(closed symbols)* and 75 mM *(open symbols)* phosphate concentration

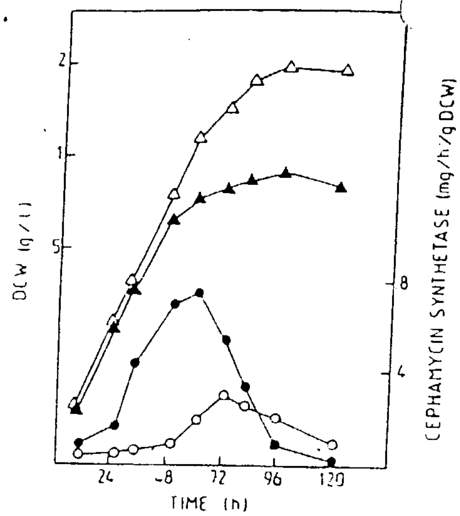

Fig. 2. Time course of specific activity of expandase (●, ○) and growth (▲, △) observed during fermentations at phosphate concentrations: 2 mM *(closed symbols)* and 75 mM *(open symbols)*

Repression of expandase biosynthesis by phosphate during fermentation

Expandase activity was measured as a function of the physiological age of the culture in two fermentations, one with phosphate as the limiting factor (2 mM phosphate) and the other with glycerol as the limiting factor (75 mM phosphate). The results obtained show a strong repression of the biosynthesis of this enzyme in the presence of 75 mM phosphate (Fig. 2).

The maximal specific activity in fermentation with phosphate as the limiting factor was reached when the specific rate was 0.025 h^{-1}.

This activity falls rapidly after 72 h whereas growth has not yet come to an end. In cells developing in the presence of 75 mM phosphate concentration (glycerol limiting), the maximum expandase biosynthesis was reached after 72 h of fermentation, at a growth rate of about 0.016 h^{-1}. The biosynthesis of this enzyme is rather late due probably to a derepression by glycerol exhaustion.

Repression of the biosynthesis of enzymatic systems producing cephamycin and clavulanic acid by phosphate during fermentation

In order to show the effect of excess phosphate on the biosynthesis of the enzymatic systems responsible for the production of cephamycin and clavulanic acid during fermentations with 2 mM and 75 mM phosphate concentrations, regular sample collections were made from cells which were used for the preparation of resting cells in the presence of streptomycin. The resting cells were incubated at 28°C for 6 h; during this period cephamycin and clavulanic acid were linearly produced, which allowed a calculation of the activity of these two enzymatic systems.

The results (Figs. 3, 4) show that in the presence of 2 mM phosphate the specific activity of these two systems is maximal when the specific

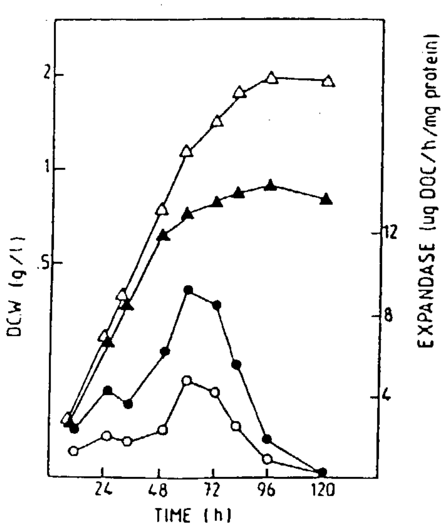

Fig. 3. Time course of specific activity of "cephamycin synthetase" (●, ○) and growth (▲, △) observed during fermentations at phosphate concentrations: 2 mM *(closed symbols)* and 75 mM *(open symbols)*. Activity was determined with RCS in the presence of 100 μg/ml streptomycin

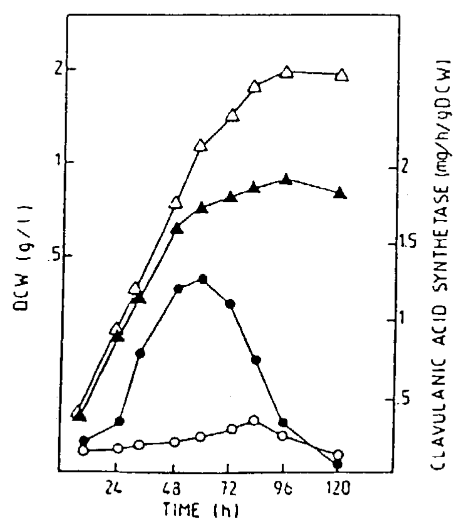

Fig. 4. Time course of specific activity of "clavulanic acid synthetase" (●, ○) and growth (▲, △) observed during fermentations at phosphate concentrations: 2 mM *(closed symbols)* and 75 mM *(open symbols)*. Activity was determined with RCS in the presence of 100 μg/ml streptomycin

growth rate is between 0.025 and 0.006 h^{-1}. Afterwards the specific activity falls rapidly, stopping completely after 120 h of fermentation. This maximal specific activity coincides with the maximal specific rates of production of clavulanic acid and cephamycin. When phosphate concentration is 75 mM, the specific activities of these two systems are much lower, due to their repression by excess phosphate. The specific activity of these two systems is maximal when the specific growth rate, limited by glycerol exhaustion, is between 0.014 and 0.008 h^{-1}.

Effect of initial inorganic phosphate concentrations on the biosynthesis of cephamycin synthetase

The cells were cultivated at different phosphate concentrations, recovered by filtration after 48 h of fermentation, washed with a MOPS buffer solution (10 mM at pH 6.9, containing 100 mg/l streptomycin) and used for the preparation of the resting cells. The specific activity of this enzymatic system is maximal in cells grown in the presence of 10 mM phosphate, whereas the cultivation of cells in the presence of 100 mM provokes a reduction in the specific activity to about 75% in comparison to the specific maximal activity (Fig. 5).

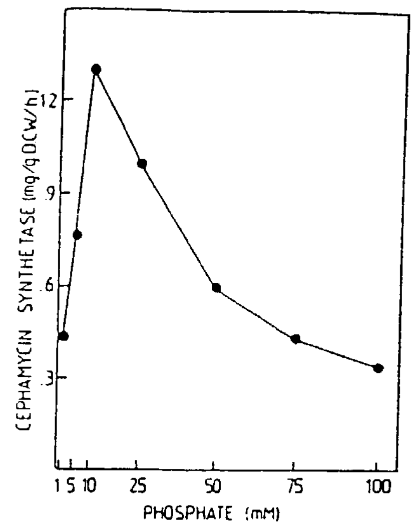

Fig. 5. Effect of initial concentrations of phosphate in growth medium on the specific activity on cephamycin synthetase

Effect of initial inorganic phosphate concentrations on the biosynthesis of expandase

Cells were cultivated at different phosphate concentrations, recovered by filtration after 48 h of fermentation, washed with distilled water and suspended in the buffer solution.

The expandase activity was measured in cell-free extracts prepared by sonication. The specific expandase activity was maximal in cells grown in the presence of 1 mM phosphate. As soon as the cells are exposed to even slightly higher concentrations in the fermentation medium, biosynthesis of expandase is drastically repressed; 20 and 100 mM phosphate caused a reduction in the specific activity of about 50% and 80% respectively (Fig. 6).

Expandase as an important enzyme

A comparison of Figs. 5 and 6 shows that there is a correlation between the specific expandase activity and that of cephamycin synthetase (Fig. 7). This correlation does not take into consideration the enzymatic activities of cephamycin synthetase at phosphate concentrations lower than 10 mM. It reveals the important role of expandase in the limitation of production of β-lactams by phosphate. These results confirm the results reported by Lubbe et al. (1985), which show that the biosynthesis of cyclase and epimerase is not influenced by repressor effects of phosphate.

Discussion

Amongst the elements added to fermentation media used in secondary metabolite production,

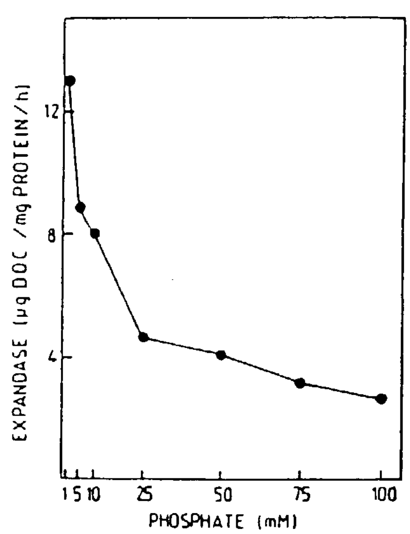

Fig. 6. Effect of initial concentrations of phosphate in growth medium on the specific activity of expandase

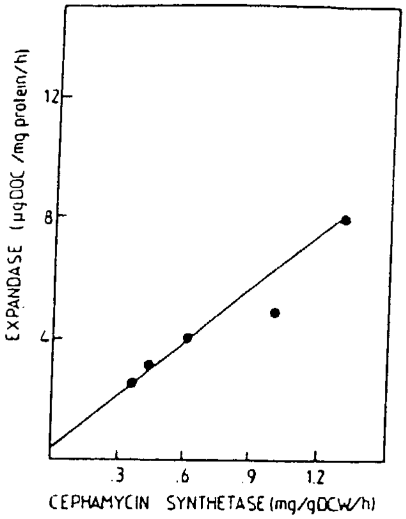

Fig. 7. Correlation between the expandase specific activity and cephamycin synthetase specific activity at phosphate concentrations higher than 10 mM

phosphate is one of the most critical (Weinberg 1974). Many antibiotics are only produced at concentrations of inorganic phosphate suboptimal for growth. This effect is manifested by several antibiotics-producing *Streptomyces* (Martin 1977). Phosphate in the range of 0.3 to 500 mM permits excellent cell growth, whereas 10 mM phosphate often suppresses biosynthesis of antibiotics.

The negative effect of phosphate on the production of cephamycin by *S. clavuligerus* (Aharonowitz and Demain 1977), streptomycin by *S. griseus* (Inoue et al. 1982), and thienamycin by *S. cattleya* (Lilley et al. 1981) has been demonstrated. We have shown that increasing phosphate concentrations in the fermentation medium provokes a drop in cephamycin C biosynthesis. Analysis of the specific activity of the cephamycin synthetase system after culture at different phosphate concentrations shows that phosphate represses the biosynthesis of one or several enzymes of the pathway involved in the production of cephamycin C. This repressor effect of phosphate affects the synthesis of expandase, as is shown by analysis of the specific activity of this enzyme after culture at different phosphate concentrations.

At phosphate concentrations greater than 10 mM, we have established a correlation between the repression by phosphate of the synthesis of expandase and the repression of cephamycin synthetase. At phosphate concentrations below 10 mM such a correlation cannot be established because expandase is not limiting. The results show that expandase plays an important role in the regulation of cephamycin C biosynthesis by *S. clavuligerus* at phosphate concentrations greater than 10 mM.

Our observations complement the results obtained by Lubbe et al. (1985), who showed that, unlike expandase, cyclase and epimerase are not repressed by excess phosphate. Expandase and cyclase activities are inhibited by phosphate, but the inhibition is weaker for cyclase than for expandase.

Romero et al. (1984) demonstrated the negative effect exercised by excess phosphate on the production of clavulanic acid by *S. clavuligerus*. At that stage, the mechanism of the action of phosphate on the production of clavulanic acid was not understood. Our results confirm those of Romero et al. (1984) and show that phosphate controls the biosynthesis of clavulanic acid by a repression of the clavulanic acid synthetase enzymatic system.

Besides its effect on expandase, phosphate may also control δ-(α-aminoadipyl) cysteinyl valine synthetase (the first enzyme in the biosynthetic pathway). This is at present being investigated in our laboratory.

References

Aharonowitz Y, Demain AL (1977) Influence of inorganic phosphate and organic buffers on cephalosporin production by *Streptomyces clavuligerus*. Arch Microbiol 115:169—173

Aharonowitz Y, Demain AL (1978) Carbon catabolite regulation of cephalosporin production in *Streptomyces clavuligerus*. Antimicrob Agents Chemother 14:159—164

Bok HS, Demain AL (1977) An improved colorimetric assay for polyols. Anal Biochem 81:21—27

Brana AF, Wolfe S, Demain AL (1985) Ammonium repression of cephalosporin production by *Streptomyces clavuligerus*. Can J Microbiol 31:736—743

Brown AG, Butterworth D, Cole M, Hauscomb G, Hodd JD, Reading C (1976) Naturally-occurring β-lactamase inhibitors with antibacterial activity. J Antibiot (Tokyo) 29:668—669

Higgens CE, Kastner RE (1971) *Streptomyces clavuligerus* sp., a new β-lactam antibiotic producer. Int J Syst Bactiol 21:326—331

Hu WS, Brana AF, Demain AL (1984) Carbon source regulation of cephem antibiotic production by resting cells of *Streptomyces clavuligerus* and its reversal by protein synthesis inhibitors. Enzyme Microbiol Technol 6:155—160

Inoue S, Nishizawa Y, Nagai S (1982) Stimulation of streptomycin formation by *Streptomyces griseus* grown in a phosphate deficient culture. J Ferment Technol 60:417—422

Jensen SE, Westlake DWS, Wolfe S (1983) Analysis of penicillin N ring expansion activity from *Streptomyces clavuligerus* by ion-pair high pressure liquid chromatography. Antimicrob Agents Chemother 24:307—312

Lilley G, Clark AE, Lawrence GC (1981) Control of the production of cephamycin C and thienamycin by *Streptomyces cattleya* NRRL 8057. J Chem Technol Biotechnol 31:127—134

Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951) Protein measurement with the Folin phenol reagent. J Biol Chem 193:265—273

Lubbe C, Wolfe S, Demain AL (1985) Repression and inhibition of cephalosporin synthetases in *Streptomyces clavuligerus* by inorganic phosphate. Arch Microbiol 140:317—320

Martin JF (1977) Control of antibiotic synthesis by phosphate. Adv Biochem Eng 6:105—127

Nagarajan R (1972) β-lactam antibiotics from *Streptomyces*. In: Flynn EH (ed) Cephalosporins and penicillins, chemistry and biology. Academic, New York, pp 636—661

Romero J, Liras P, Martin JF (1984) Dissociation of cephamycin and clavulanic acid biosynthesis in *Streptomyces clavuligerus*. Appl Microbiol Biotechnol 20:318—325

Weinberg ED (1974) Secondary metabolism: control by temperature and inorganic phosphate. Dev Ind Microbiol 15:70—81

Received July 28, 1986/Revised October 28, 1986